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Chemokine and Chemokine-Receptor Expression in Human Glial Elements

Induction by the HIV Protein, Tat, and Chemokine Autoregulation

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Human immunodeficiency virus (HIV) encephalitis is a prominent pathology seen in children infected with HIV. Immunohistochemical analyses of pediatric brain tissue showed distinct differences in expression of C-C chemokines and their receptors between children with HIV encephalitis and those with non-CNS-related pathologies. Evidence suggests that soluble factors such as HIV Tat released from HIV-infected cells may have pathogenic effects. Our results show Tat effects on chemokines and their receptors in microglia and astrocytes as well as chemokine autoregulation in these cells. These results provide evidence for the complex interplay of Tat, chemokines, and chemokine receptors in the inflammatory processes of HIV encephalitis and illustrate an important new role for chemokines as autocrine regulators. ***(Am J Pathol 2000, 156:1441–1453)***

Chemokines are small molecular weight proteins involved in the processes of leukocyte recruitment and activation.[1,2] There are several families of chemokines classified by the position of their N-terminal cysteines; C-X-C, C-C, C, and C-X_3-C. The C-C chemokine family, including monocyte chemoattractant protein 1 (MCP-1) and the macrophage inflammatory proteins (MIP-1α and MIP-1β), serves primarily as chemoattractants for monocytes and T cells. These proteins function through binding of specific seven transmembrane domain spanning, G-protein-coupled receptors. These receptors bind chemokines within their family and the C-C chemokine receptor family is continually growing with approximately 10 receptors identified. Chemokine receptor binding within each family is somewhat promiscuous with MIP-1α binding CCR1 and CCR5, MIP-1β binding CCR5, and MCP-1 using the CCR2 receptor. Both chemokines and their receptors have been shown to play key roles in human immunodeficiency virus (HIV) infection and progression. Several chemokine receptors are co-factors with CD4 for the entry of HIV into host cells, the major receptors being CCR5 and CXCR4.[3–6] Chemokines have been shown to compete with HIV for binding of chemokine receptors and as such may play a role in controlling the spread of the virus within the host.[7]

A major complication of HIV infection, particularly in children, is encephalitis with approximately one-third of those infected with HIV developing HIV encephalitis and/or acquired immune deficiency syndrome dementia complex.[8] Although much is known about the role of chemokines and their receptors in the pathogenesis of HIV infection, little is known of their role in HIV infection of the central nervous system (CNS) and the neural complications which result.[9,10] Thus, it is critical to determine the expression and regulation of chemokines and their receptors in the CNS and how this is affected by HIV infection. Chemokine receptors are expressed constitutively in the CNS whereas chemokines are rarely detected in normal CNS but are highly expressed during a variety of CNS pathologies. We and others have demonstrated the expression of chemokines in the CNS in inflammatory pathologies including MIP-1α, MIP-1β, MCP-1, MCP-2, and MCP-3[11–14] and several recent reports demonstrate the expression of various chemokine receptors in the CNS.[9,15–18] CXCR4 has been shown to be expressed on astrocytes, microglia, and neurons as has CCR5 in normal CNS (reviewed in Ref. 19). In this report, we analyze tissue sections from brains of pediatric acquired immune deficiency syndrome patients, with and without encephalitis, as well as aged-matched normal

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Table 1. Immunocytochemical Studies

Case	HIV status	Age/sex	Brain pathology
1	+	1 y/F	HIV encephalitis, HIV leukoencephalopathy
2	+	6.5y/M	HIV encephalitis, calcific vasculopathy
3	+	6 y/M	HIV encephalitis, multiple encephalomalacia
4	+	9 wk/F	HIV encephalitis, calcific vasculopathy
5	+	2.5 y/M	HIV encephalitis, HIV leukoencephalopathy, calcific vasculopathy
6	+	14 wk/F	Focal chronic meningitis
7	+	5.5 mo/M	Metabolic gliosis, calcific vasculopathy
8	−	16 mo/F	Normal brain
9	−	1 y/M	Normal brain
10	−	2 y/M	Normal brain

control tissue for the expression of the C-C chemokines, MIP-1α, MIP-1β, and MCP-1 and the chemokine receptors CCR2, CCR5, and CXCR4.

Microglia have been shown to be the primary productively infected cell type of the CNS[8,20] whereas astrocyte infection, although reported, is controversial.[21,22] Levels of virus in the CNS do not always correlate with neurological dysfunction and microglial activation is common in areas of the CNS where HIV antigen is not present.[23] Thus, soluble factors released from HIV-infected cells may have effects on uninfected cells. Tat, an HIV transactivator protein, is secreted from HIV-infected cells[24–26] by a leaderless pathway.[27] Little is known about the *in vivo* effects of this extracellular protein, particularly within the CNS. However, a recent report by Jones and colleagues[28] shows that intraventricular injection of Tat into male rats results in ventricular enlargement, apoptosis, and inflammation. Evidence for the expression of Tat within the CNS is reported[29,30] and Tat has also been detected in the serum of patients infected with HIV.[31] There is a growing literature on the *in vitro* effects of Tat. Tat has been shown to mimic certain properties of C-C chemokines[32] and can up-regulate CXCR4 on resting $CD4^+$ T cells.[33] With regards to the CNS, data indicates that Tat induces cytokine and adhesion molecule expression by brain microvascular endothelial cells as well as glial cells.[34–36] It has been reported to have potent neurotoxic effects[27,37] and a recent report by Conant and colleagues[38] showed that Tat can induce MCP-1 in astrocytes. For this study, we analyzed the effects of the HIV protein, Tat, on chemokine and chemokine receptor expression in human fetal astrocytes and microglia.

We have shown previously that astrocytes and microglia produce C-C chemokines in response to proinflammatory cytokines.[39] Cytokines are major mediators of the inflammatory response serving to activate cells and mediate host responses. Chemokines are present in the local environment of the inflammatory response and studies indicate an essential role for chemokines in the establishment of this response because several studies have shown that blocking chemokine expression can ameliorate inflammatory disease.[40,41] Chronic neurological dysfunction can result in part from a continuing inflammatory response. How this response is perpetuated is not clearly understood. We determined whether chemokines could act in an autocrine fashion to induce their own expression, and thus, play a role not only in the inflammatory response but also in the perpetuation of this response. We analyzed the pattern of expression of chemokines and the C-C chemokine receptors, CCR1-CCR5, in response to chemokine treatment of human fetal astrocytes and microglia and show a novel autocrine function for chemokines.

Materials and Methods

Tissue Collection and Preparation

Immunocytochemical studies were performed on brain tissue taken at autopsy. Seven pediatric patients with HIV encephalitis were studied along with nonencephalitic brains from two patients with HIV infection and three age-matched control brains (Table 1). Astroglioma tissue and lung tissue from patients with pneumonia were used as positive controls because these tissues are known to express high levels of chemokines.

Antibodies

Primary antibodies for use in immunohistochemistry were obtained from the following sources: MCP-1, MIP-1α, and MIP-1β were kindly provided by Leukosite Inc. (Cambridge, MA). MCP-1 and MIP-1α are purified monoclonal antibodies used at a concentration of 2.8 μg/ml and MIP-1β was an ascites used at a dilution of 1:250. All antibodies were screened by Leukosite for reactivity to chemokines on paraffin-embedded tissues. CCR2, CCR5, and CXCR4 antibodies were kindly provided by Berlex Biosciences (San Francisco, CA) and used at a concentration of 5 μg/ml. Isotype-matched antibodies, IgG1, IgG2a, and IgG2b were purchased from Cappel (Durham, NC) and used as a negative control at a concentration of 2.8 μg/ml.

Figure 1. Immunohistochemical analysis of chemokine expression in HIV encephalitis. Immunohistochemistry of both HIV encephalitic pediatric brain tissue and normal control tissues. **A** and **B:** Staining with MIP-1α. The tissue in **A** is from an encephalitogenic HIV-infected individual. **B:** The lack of immunoreactivity of normal brain tissue for MIP-1α. **C** and **D:** Staining of MIP-1β in HIV encephalitis both in the parenchyma of the tissue (**C**) and surrounding a vessel (**D**). MCP-1 expression in HIV encephalitis is shown in **E** and **F**. This illustrates reactivity of the endothelium (**E**) and astrocytes and microglia in the parenchyma (**F**). **G:** A normal brain stained for MCP-1. **H:** a representative isotype matched control for these antibodies. Magnification: **A–H,** ×82.5.

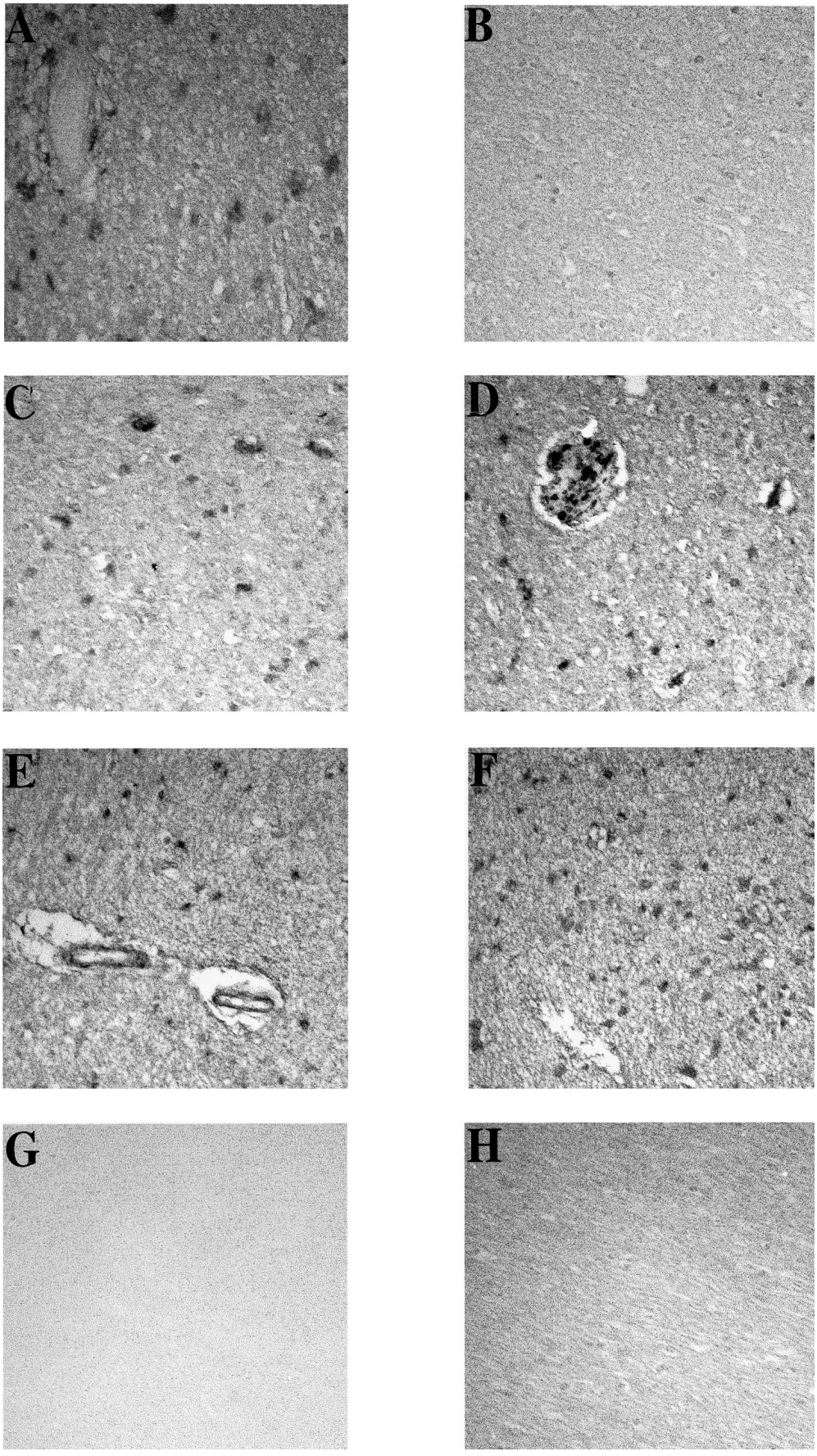
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Table 2. Immunoreactivity of Chemokines and Chemokine Receptors in HIV Encephalitis

	Astrocytes	Microglia	Endothelium	PBMCs	Neurons
Chemokines					
MIP-1α	+++	+++	−	++	−/+
MIP-1β	+++	+++	−	++	−/+
MCP-1	+++	++	++	++	+
Chemokine receptors					
CCR2	+	+	−	+	−
CCR5	++	++	−	++	+
CXCR4	+++	+	++	++	+++

Immunoreactivity was determined on an arbitrary scale; −, no reactivity; +++, extensive reactivity.

Immunohistochemistry

Paraffin-embedded tissue was dehydrated in graded alcohol baths and then deparaffinized in xylene. After rehydration the sections were quenched for 20 minutes in 0.8% hydrogen peroxide in methanol. Sections were then incubated in 2% normal horse serum (Vector Laboratories, Burlingame, CA) followed by an overnight incubation at 4°C in primary antibody. The sections were washed and incubated with a biotinylated secondary antibody (1:750; Vector Laboratories) followed by incubation in avidin-biotin-peroxidase complex (Vector Laboratories). The slides were developed with 3′3′-diaminobenzidene to give a brown reaction product (Sigma, St. Louis, MO) and then dehydrated and mounted with Cytoseal (VWR, Boston, MA).

Immunoelution of Tat

Protein A agarose beads were pelleted and washed and purified rabbit anti-Tat (1:50 dilution) was added and incubated for 1 hour at room temperature. After washing, Tat was added at the treatment concentration for 1 hour at room temperature. After centrifugation, the supernatant was used in the enzyme-linked immunosorbent assay (ELISA) studies.

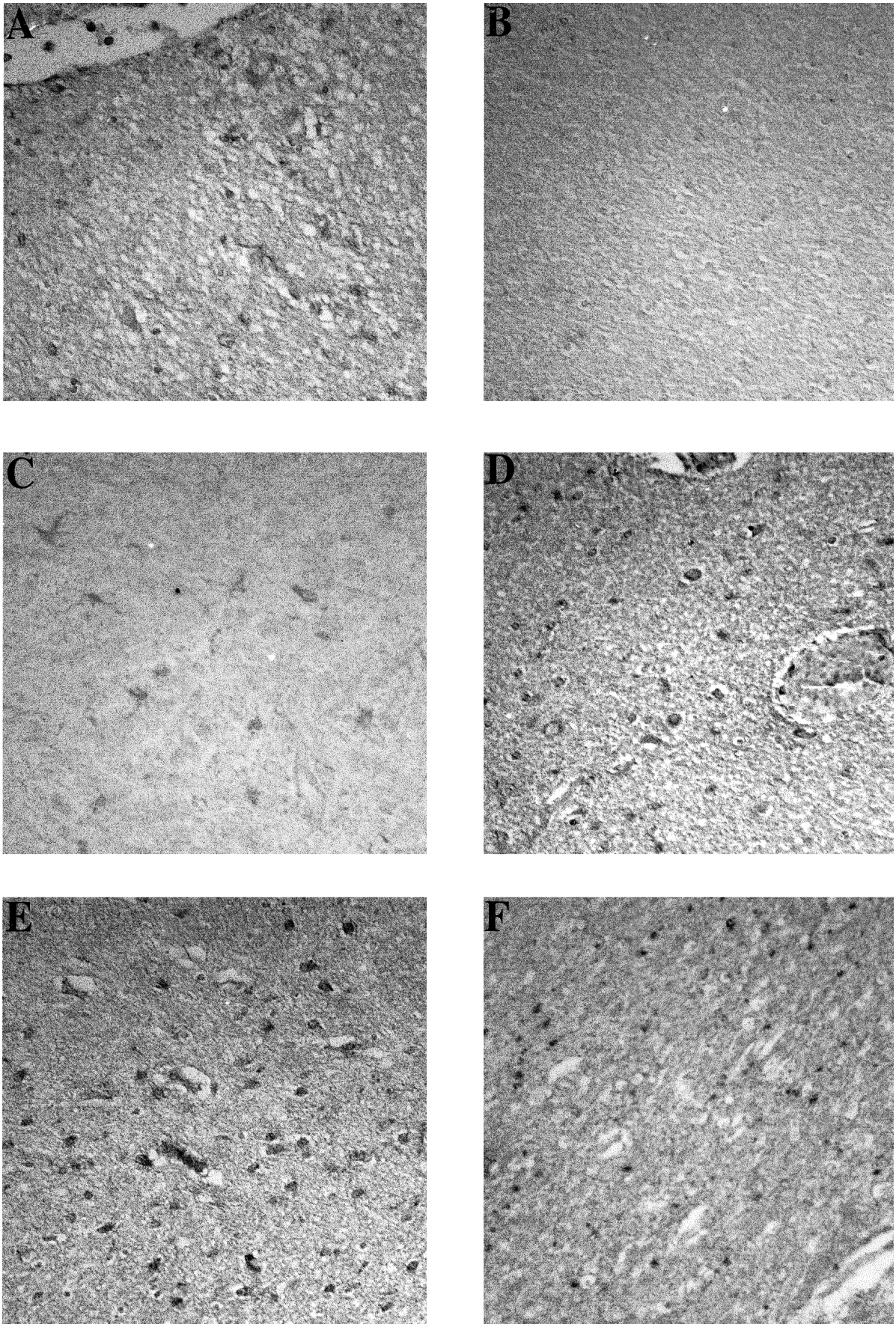
Cell Culture and Reagents

Human fetal CNS tissue (20 to 23 weeks) was obtained at the time of elective termination of intrauterine pregnancy from otherwise healthy females. Informed consent was obtained from all participants. This tissue was used as part of an ongoing research protocol that has been approved by the Albert Einstein College of Medicine Committee on Clinical Investigation and the City of New York Health and Hospitals Corporation. Microglia and astrocyte cultures were prepared as previously described.[39,42] Briefly, tissue was dissociated and incubated for 45 minutes at 37°C in 1× Hanks' balanced salt solution (Life Technologies, Inc., Grand Island, NY), 1× trypsin (Life Technologies, Inc.), and DNase 1 (Boehringer-Mannheim, Indianapolis IN). Tissue fragments were passed through 250- and 150-μm nylon mesh (Tetko, Inc., Briar Cliff Manor, NY). Cells were washed and resuspended in complete Dulbecco's modified Eagle's medium (25 mmol/L HEPES, 10% fetal calf serum, 1% nonessential amino acids, and 1% penicillin-streptomycin) and rewashed. Cells were seeded at 1.2×10^8 cells per 150-cm^2 tissue-culture flask (Falcon; Becton Dickinson, Franklin Lakes, NJ) and cultured for 12 days. Microglial cells were then removed from the mixed culture by shaking for 30 minutes at 4°C and plated in complete Dulbecco's modified Eagle's medium at a concentration of 1×10^6 cells per 20-cm^2 tissue-culture plate (Falcon). Cells were analyzed for the purity of the culture and shown to be ≥95% HAM56 (a microglial marker) positive. Astrocytes, the adherent population remaining in the 150-cm^2 flask, were placed in RPMI 1640 medium with 10% fetal calf serum and 1% penicillin-streptomycin. These cells were then passaged several times and allowed to grow to confluency. The astrocyte cultures were ≥95% GFAP-positive (an astrocyte marker). Cells were treated with human recombinant MIP-1α, MIP-1β at 1 to 100 ng/ml (R&D Systems), MCP-1 at 1 to 100 ng/ml (Pharmingen, San Diego, CA), or HIV Tat protein at 1 to 100 ng/ml (prepared as previously described[43]). Briefly, the recombinant Tat was prepared by expressing the *tat* gene encoding amino acids 1 to 72 (first exon) as a fusion protein in *Escherichia coli* DH5αf'1Q (Life Technologies, Inc.) and purifying it with a metal chelate affinity column. Tat was diluted with the following buffer before use: 50 mmol/L Tris, pH 8.0; 100 mmol/L NaCl; 1 mmol/L $CaCl_2$; 0.5 mmol/L dithiothreitol. Endotoxin levels for all of the chemokines and the Tat were <1 ng/ml as tested by Limulus assay (BioWhitaker, Walkersville, MD). To accurately reflect donor variation each donor is reported as a separate symbol in all figures. Cell numbers obtained from each donor also varied due to tissue sample size and cell recovery, accounting for differences in numbers of untreated *versus* treated conditions.

RNA Extraction and RNase Protection Assay

Total RNA was extracted from microglial cultures using Tri-Reagent (Molecular Research Center, Cincinnati, OH).

Figure 2. Immunohistochemical analysis of chemokine receptor expression. Immunohistochemistry illustrating the expression of chemokine receptors in pediatric HIV encephalitis. CCR2 is expressed in HIV encephalitis (**A**) but is minimally detectable in normal brains (**B**). CCR5 immunoreactivity was seen on neurons, astrocytes, microglia, and mononuclear cells (**C** and **D**). This receptor is seen in both normal (**D**) and encephalitic brains (**C**). Similar to CCR5, CXCR4 is expressed in normal brain (**F**) and HIV encephalitis (**E**) but appears to be more intense in encephalitogenic tissues (**E**). Magnification: **A–F,** ×82.5.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Probe cocktails of C-C chemokines (panel CR5), C-C chemokine receptors (panel CK5), and C-X-C chemokine receptors (panel CR6) were obtained from Pharmingen. Ambion Maxiscript kit was used according to manufacturer's instruction for the generation of the RNA probes and the Ambion RPA II kit was used according to manufacturer's instructions for the analysis of mRNA expression. Samples were analyzed by 5% denaturing acrylamide gels followed by autoradiography (Fischer, Springfield, NJ). Densitometry was performed on multiple film exposures using densitometric software (NIH Shareware).

Chemokine ELISA

Supernatants from microglial cell cultures were analyzed for chemokine proteins. Matched antibody pairs for MIP-1α and MIP-1β were purchased from R&D Systems (Minneapolis, MN) for use in sandwich ELISA. Antibody pairs for MCP-1 were purchased from Pharmingen. Ninety-six well plates were coated with chemokine antibody at a concentration of 4 μg/ml in 1$\times$ phosphate-buffered saline (PBS) at 4°C overnight. Plates were then washed with 1$\times$ PBS with 0.005% Tween-20 (Bio-Rad, Hercules, CA) and blocked with 1% bovine serum albumin in PBS. Samples were then added to the plate and a standard curve was generated using a series of dilutions from 2000 pg/ml to 31.25 pg/ml of the appropriate recombinant human chemokine (MIP-1α, MIP-1β, R&D Systems; MCP-1, Pharmingen). Samples and standards were allowed to incubate overnight at 4°C. Plates were then washed again and incubated for 1 hour at room temperature with specific biotinylated secondary antibody, washed, and incubated for 30 minutes in avidin-peroxidase (Sigma). The plates were washed, TMB (KPL, Gaithersburg, MD) substrate added for 5 minutes or less and the reaction stopped with 1 mol/L phosphoric acid. Absorbance was read at 450-nm wavelength on a microplate reader (Bio-Rad) within 30 minutes of stopping the reaction.

Statistical Analyses

Statistical significance for microglia and astrocyte chemokine protein expression was determined using the Wilcoxon signed rank test. This test is nonparametric and as such analyzes each experimental group individually, allowing for an accurate measurement of variability in chemokine induction. This test does not generate error bars because the *P* value is not based on the means of pooled experiments. Individual points are represented in the graphs to illustrate the variability between experiments. Densitometric results of the RNase protection assays were analyzed using the student's paired *t*-test. Significance, with both analyses, was assigned for $P \leq 0.05$ (StatView; Abacus Concepts, Berkeley, CA).

Results

Immunohistochemical Analysis of MIP-1α, MIP-1β, and MCP-1 Expression

Staining of pediatric tissues showed that chemokines are highly expressed in the brains of children with HIV encephalitis but are not constitutively expressed in the brains of children with non-CNS-related pathologies. Figure 1A shows MIP-1α staining of a highly encephalitogenic case of HIV encephalitis where immunoreactivity is noted in the parenchyma by glial cells. Figure 1B, an age-matched control tissue with non-CNS-related pathologies, is negative for MIP-1α staining and is representative of staining of normal tissue with MIP-1β (data not shown). Figures 1C and 1D illustrate MIP-1β expression in HIV encephalitis particularly around blood vessels by glial cells and perivascular mononuclear cells (Figure 1D) and in the nonvessel-associated white matter (Figure 1C). Staining for MIP-1β is intense on astrocytes as well as microglia and, similar to MIP-1α, staining is seen on mononuclear cells infiltrating the CNS. MCP-1 is expressed by astrocytes, microglia, and mononuclear cells as well as on the endothelium (Figure 1, E and F). Figure 1G illustrates the lack of MCP-1 immunoreactivity in age-matched control tissue. Figure 1H is an isotype-specific negative control reagent that is nonreactive in the tissues analyzed and is representative of all isotype-matched controls. A summary of the immunohistochemical results is shown in Table 2.

Immunohistochemical Analysis of CCR2, CCR5, and CXCR4 Expression

Analyses of chemokine receptor expression in pediatric HIV encephalitis demonstrated that the expression of these receptors is not always restricted to disease states, in that some of the receptors were also expressed in normal brains. CCR2 was not detected in the parenchyma of normal brains (Figure 2B), although it was expressed on glial cells and peripheral mononuclear cells within vessels in HIV encephalitis (Figure 2A). This differs from what was seen with CCR5 and CXCR4 which were present in both normal (Figure 2, D and F) and HIV encephalitogenic brains (Figure 2, C and E). This correlates with recent data showing immunoreactivity for CCR5 by glial cells and mononuclear cells with staining being more intense in the encephalitic cases.[16] Anti-CXCR4 stained neurons as well as glial cells and mononuclear cells in encephalitogenic brains and in normal brains (Figure 2, E and F), again with more intense staining noted in the encephalitic brains which confirms previous data by Vallat et al.[16] Most of the sections analyzed were from the cortex and cerebellum. There were variations in the intensity of staining observed with both chemokines and chemokine receptors between different individuals, and the areas of tissue chosen for illustration are most representative of the intensity of staining seen.

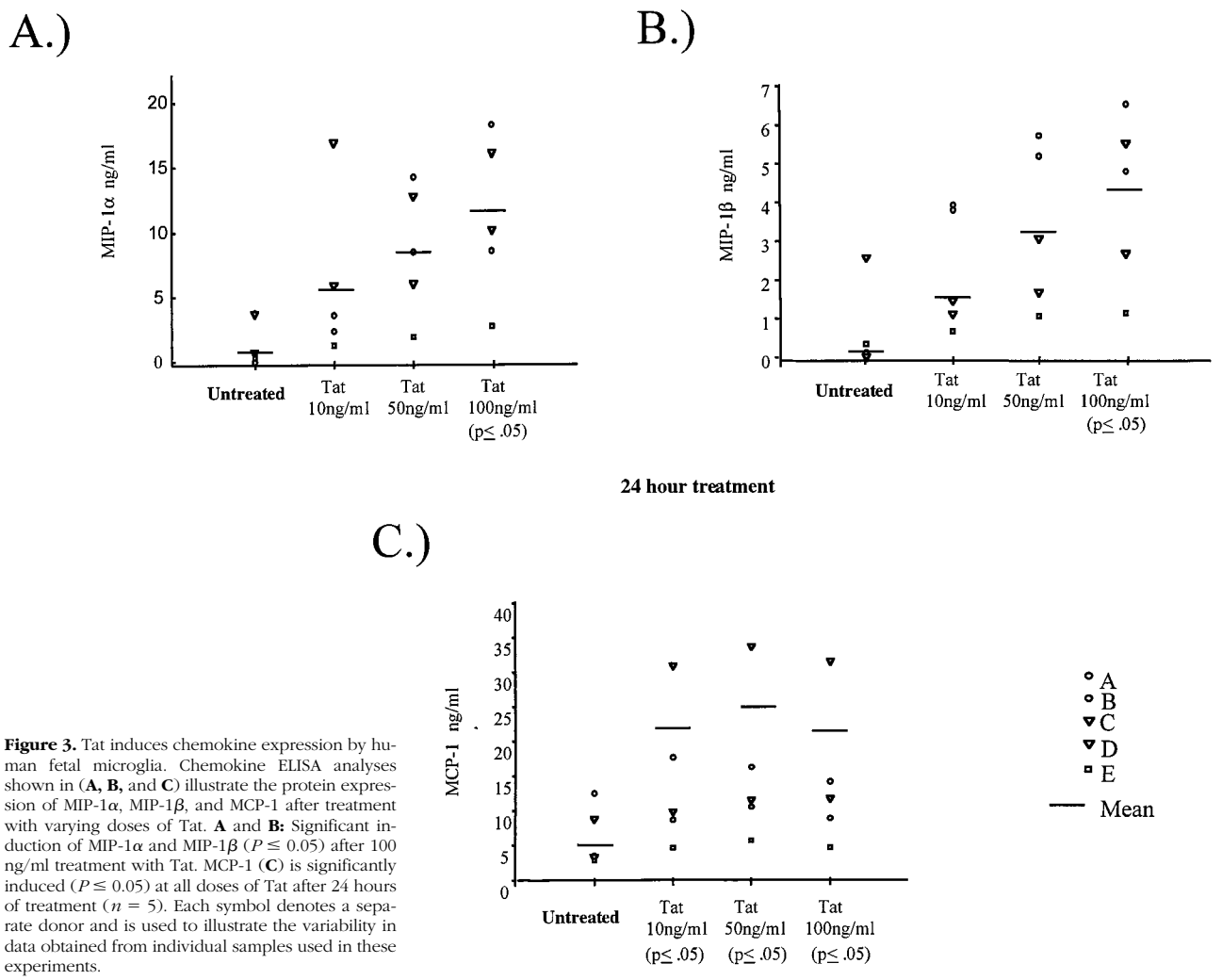

Figure 3. Tat induces chemokine expression by human fetal microglia. Chemokine ELISA analyses shown in (**A, B,** and **C**) illustrate the protein expression of MIP-1α, MIP-1β, and MCP-1 after treatment with varying doses of Tat. **A** and **B:** Significant induction of MIP-1α and MIP-1β ($P \leq 0.05$) after 100 ng/ml treatment with Tat. MCP-1 (**C**) is significantly induced ($P \leq 0.05$) at all doses of Tat after 24 hours of treatment ($n = 5$). Each symbol denotes a separate donor and is used to illustrate the variability in data obtained from individual samples used in these experiments.

Effects of the HIV Protein, Tat, on Human Fetal Microglia and Astrocytes

Recent reports suggest an important role for Tat in modulating the cellular activation of glial cells and in contributing to the pathogenesis of HIV encephalitis. Much of the damage seen in HIV encephalitis may not be correlated with viral load and thus may be mediated by HIV proteins, such as Tat, which are secreted by HIV-infected cells. We examined the effects of Tat on chemokine and chemokine receptor expression in the CNS.

O-RNase Protection Assay (RPA) analysis shows induction of MIP-1α, MIP-1β, and MCP-1 mRNA after treatment with Tat (10 ng/ml) ($n = 3$; data not shown). Treatment of microglia for 24 hours with varying doses of Tat results in significant up-regulation of both MIP-1α, MIP-1β, and MCP-1 protein (Figure 3, A–C) as measured by ELISA. This induction is dose-dependent with MIP-1α and MIP-1β showing induction at 10 ng/ml and 50 ng/ml, with significant induction being reached after 100 ng/ml Tat treatment ($P \leq 0.05$). Tat induced higher levels of MIP-1α than MIP-1β (note ng/ml scale in Figure 3, A and B). MCP-1 is constitutively expressed and as seen previously with cytokine induction, induced to higher levels than MIP-1α or MIP-1β.[39] MCP-1 is regulated differently from MIP-1α and MIP-1β in that it is significantly induced at all doses analyzed after 24 hours of treatment ($P \leq 0.05$). The Tat-induced expression of MCP-1, MIP-1α, and MIP-1β was completely abrogated by immunoelution of Tat with polyclonal-Tat antibody (1:50) coupled to agarose beads. In these experiments chemokine levels were reduced to <3 ng/ml for MCP-1 and to <1 ng/ml for both MIP-1α and MIP-1β ($n = 2$).

Our data demonstrated that Tat can significantly induce the expression of MCP-1 in human fetal astrocytes ($n = 5$, $P \leq 0.05$; see Figure 4B). This correlates with recent data showing astrocyte production of MCP-1 in response to Tat.[38] We also analyzed the ability of Tat to induce MIP-1α and MIP-1β in astrocytes. Analysis of astrocytes did not reveal the expression of MIP-1α or MIP-1β by astrocytes in response to a variety of cytokines (data not shown). This is in contrast to data showing induction of MIP-1α by treatment with combinations of proinflammatory cytokines.[44] Tat treatment (100 ng/ml) of astrocytes did result in detectable expression of these chemokines at the picogram level which was not statistically significant (Figure 4A). RPA analysis of mRNA expression of these chemokines shows induction of MCP-1

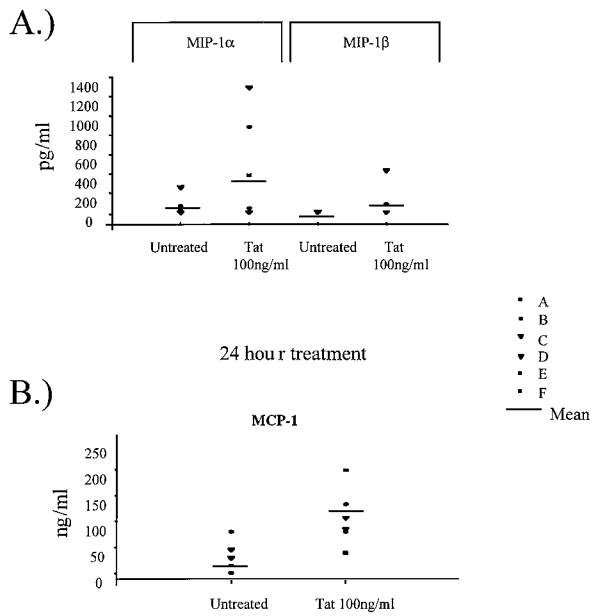

Figure 4. Tat induces chemokine expression by human fetal astrocytes. Analysis of protein expression for these chemokines by ELISA shows picogram amounts of MIP-1α and MIP-1β induced by 100 ng/ml of Tat ($n = 5$) (**A**). **B:** MCP-1 is significantly induced ($P \leq 0.05$) after 100 ng/ml treatment of astrocytes for 24 hours ($n = 5$). Each symbol denotes a separate donor. Individual donor data are illustrated to demonstrate donor variability in these experiments.

after all treatments whereas MIP-1α and MIP-1β mRNA were barely detectable ($n = 2$; data not shown).

Microglial C-C chemokine receptor mRNA expression was analyzed by RPA after treatment of the cells with varying doses of Tat for 24 hours (Figure 5A). Densitometric analyses show chemokine receptor mRNA expression to be unaffected by Tat treatment at all doses. There is a trend toward an inhibition of CCR2 and CCR5 after 50 ng/ml or 100 ng/ml of Tat but this is not significant ($n = 3$; Figure 5B). Astrocytes do not constitutively express any C-C chemokine receptor mRNA (CCR1–CCR5) and are not induced to express any of the C-C chemokine receptors after treatment with Tat ($n = 2$; Figure 5C).

Chemokine Treatment of Human Fetal Glial Cell Cultures

Our previous results showing chemokine production by glial cells in response to cytokines and other factors such as lipopolysaccharide[39] and in this report, Tat, indicate elements that could result in the induction of chemokines *in vivo*. Chemokine induction during an inflammatory response would result in the presence of chemokines in the local environment. Thus, we analyzed the ability of chemokines to induce their own production. The ability of chemokines to self-regulate would be a mechanism by which, after initial induction, chemokine levels could remain elevated and contribute to the maintenance of the inflammatory response.

We show that chemokines can induce their own production in human fetal microglia (Figure 6). RPA analysis of chemokine expression after treatment with MIP-1α, MIP-1β, or MCP-1 at 10 ng/ml for 24 hours shows that MIP-1α and MIP-1β induce themselves as well as MCP-1

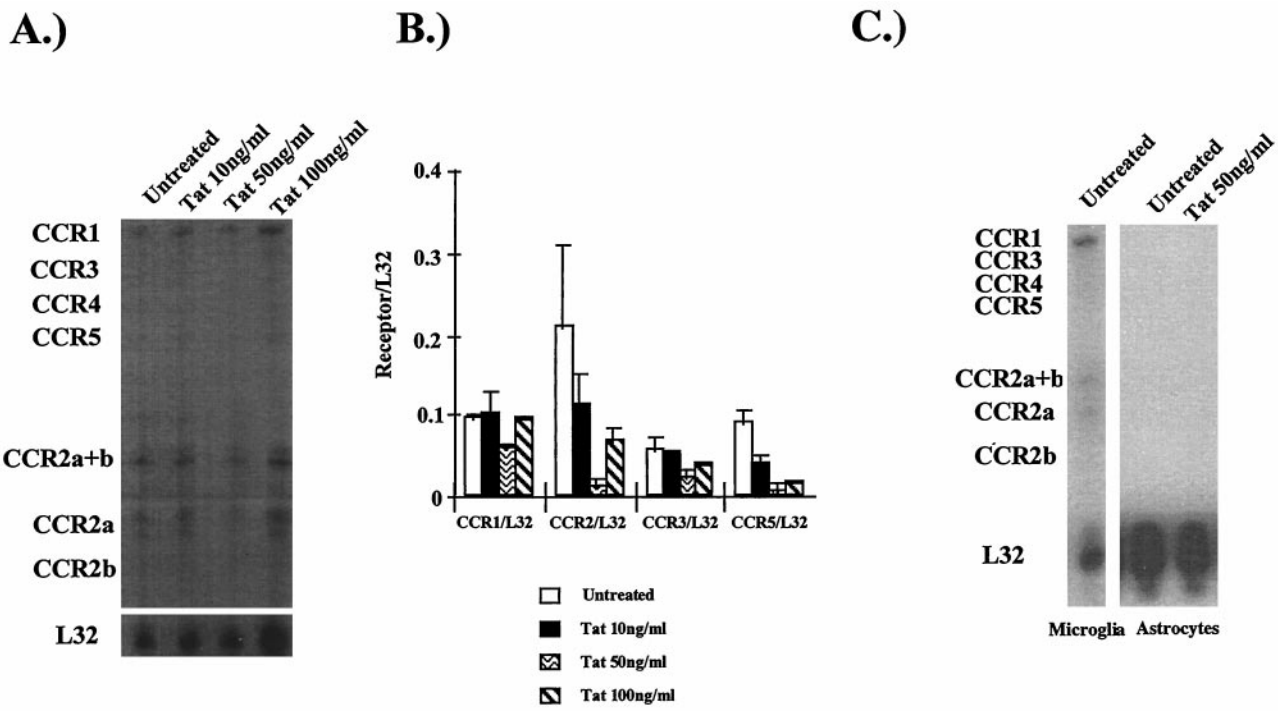

Figure 5. Tat effects on microglial and astrocyte chemokine receptors expression. RPA analysis shows the constitutive expression of the CC chemokine receptors, CCR1, CCR2, CCR3, and CCR5. This expression is not altered by the addition of varying doses of Tat for 24 hours (**A**). Densitometric analyses show no significant changes in receptor expression after Tat treatment; however, there is a trend toward Tat inhibition of CCR2 and CCR5 after 50 and 100 ng/ml treatment of Tat ($n = 3$) (**B**). RPA analysis of astrocyte chemokine receptor mRNA expression shows no constitutive expression of C-C receptors and this is not altered by treatment with Tat ($n = 2$) (**C**).

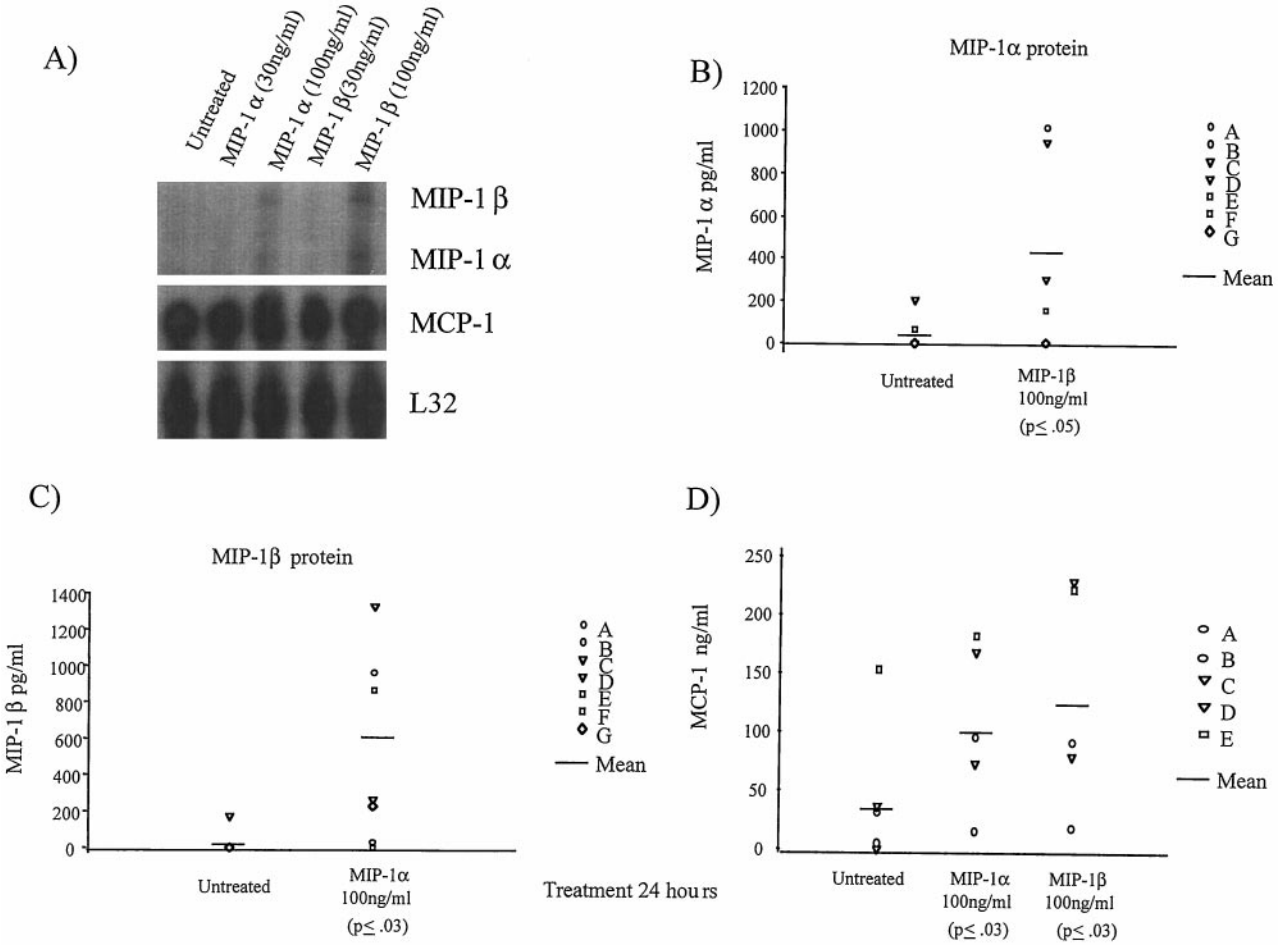

Figure 6. Chemokine effects on chemokine expression by human fetal microglia. The ability of chemokines to induce chemokine production was analyzed. ELISA analysis of cell culture supernatants shows MIP-1α and MIP-1β to induce significantly ($P \leq 0.05$) the expression of MIP-1α (**A**), MIP-1β (**B**), and MCP-1(**C**) after 24 hours of treatment ($n = 6$). Each symbol denotes a separate donor and is used to accurately demonstrate the variability in data obtained from individual donors in these experiments.

($n \geq 3$; data not shown). Protein expression of the chemokines correlated with RPA analysis. It should be noted that the MIP-1α protein expression measured after MIP-1α treatment or MIP-1β protein after MIP-1β treatment is the actual amount induced by treatment. The 10 ng/ml of protein used for treatment is subtracted giving the final numbers seen in the graphs for Figure 6. Figure 6A and 6B illustrate significant induction of MIP-1α and MIP-1β, respectively, after treatment with either chemokine (10 ng/ml) after 24 hours ($n = 6$; $P \leq 0.05$). In addition, these chemokines significantly induce the expression of MCP-1 after 24 hours ($P \leq 0.05$; Figure 6C). Interestingly, a time course study of these chemokines shows MIP-1α and MIP-1β do not significantly induce chemokines after 6 hours of treatment, but that levels remain significantly elevated after 48 hours of treatment (data not shown). MCP-1 was also analyzed for its ability to induce chemokine expression and does not induce expression of any chemokine tested as analyzed by RPA and ELISA analysis (data not shown).

Similar to what is seen for microglia, astrocytes respond to treatment with chemokines by inducing the expression of chemokines. RPA analysis of chemokine expression shows MIP-1α and MIP-1β to induce MIP-1α, MIP-1β, and MCP-1 after 100 ng/ml treatment for 6 hours ($n = 3$; Figure 7A). ELISA of cell culture supernatants after 24 hours of treatment showed that astrocytes produce pg/ml amounts of MIP-1α and MIP-1β (Figure 7, B and C) and because they were treated with ng/ml amounts of chemokine it was not possible to measure MIP-1α induction of MIP-1α protein or MIP-1β induction of MIP-1β. It is, however, clearly illustrated by the RPA in Figure 7A that MIP-α treatment of astrocytes at 100 ng/ml does induce MIP-α production. The same is seen with MIP-1β expression after treatment with MIP-1β.

Treatment of astrocytes with chemokines required much higher doses to see the significant effects detected for microglia at lower concentrations. MIP-1α induced MIP-1β and MCP-1 significantly at 100 ng/ml treatment for 24 hours and MIP-1β significantly induced MIP-1α and MCP-1 at 100 ng/ml treatment for 24 hours ($P \leq 0.05$; $n = 7$; Figure 7, B and D). A time course analysis of this induction shows that MIP-1α induces MIP-1β significantly after 6 hours of treatment but that this does not remain elevated after 48 hours in the presence of MIP-1α (data not shown). The opposite is seen with induction of MIP-

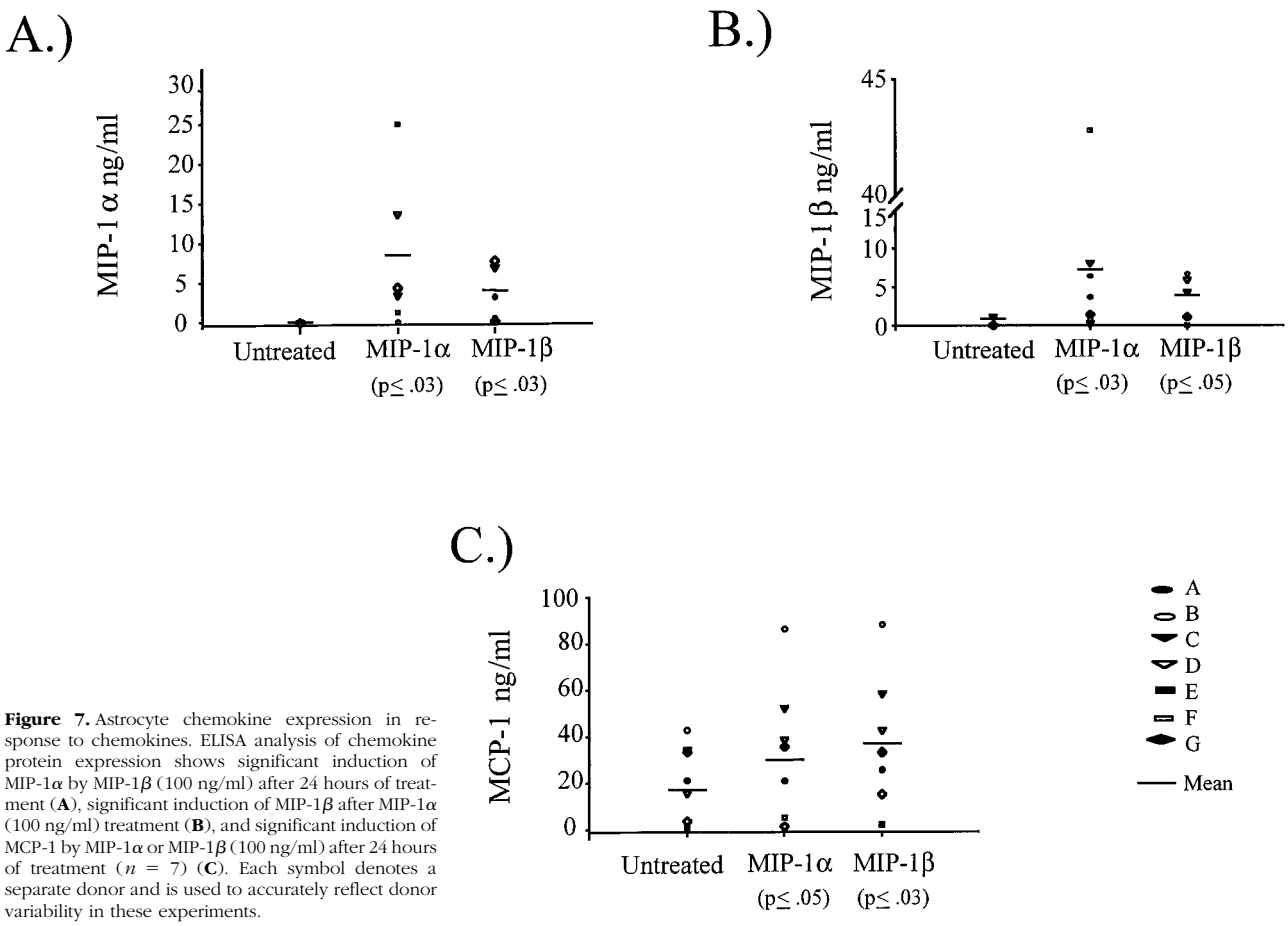

Figure 7. Astrocyte chemokine expression in response to chemokines. ELISA analysis of chemokine protein expression shows significant induction of MIP-1α by MIP-1β (100 ng/ml) after 24 hours of treatment (**A**), significant induction of MIP-1β after MIP-1α (100 ng/ml) treatment (**B**), and significant induction of MCP-1 by MIP-1α or MIP-1β (100 ng/ml) after 24 hours of treatment (n = 7) (**C**). Each symbol denotes a separate donor and is used to accurately reflect donor variability in these experiments.

1α, where MIP-1β does not significantly induce its expression by 6 hours but protein levels are still significantly elevated at 48 hours, although less than what is seen at 24 hours (data not shown). MCP-1 is significantly induced by MIP-1α at 6 hours, but not by MIP-1β, and MCP-1 remains significantly induced by both treatments after 48 hours (data not shown). As was noted for microglia, MCP-1 did not have the ability to induce chemokine expression in astrocytes (data not shown).

Treatment of microglia with chemokines did not result in significant effects on chemokine receptor mRNA expression (data not shown). This was measured by densitometric analysis of RPAs (n = 2 for MCP-1; n = 3 MIP-1α, MIP-1β). In addition, there were no effects measured on astrocyte receptor expression after chemokine treatment (data not shown).

Discussion

The data presented illustrate chemokine as well as chemokine receptor protein in the CNS of patients with pediatric HIV encephalitis as well as the first evidence of chemokine autocrine regulation in human CNS cells. The data presented show: 1) chemokines and their receptors are differentially expressed in the brains of patients with HIV encephalitis compared to nonencephalitic HIV brains and normal age-matched control brains; 2) HIV Tat protein is a strong inducer of chemokines in microglia and astrocytes; and 3) chemokines induce chemokine expression in both astrocytes and microglia.

The role of chemokines and chemokine receptors in the CNS, particularly with regards to HIV encephalitis, is still not clearly defined. The expression of chemokines and their receptors could be a consequence of the inflammation, as we have previously shown chemokines to also be highly expressed in the brains of patients with multiple sclerosis, an inflammatory autoimmune disease of the CNS.[11,12] However, this could also be a protective host response to try to contain the spread of virus within the CNS where excess chemokine production could compete with the virus for receptor binding. It is necessary to understand the complex interplay of chemokines with receptors and what cellular events, ie, signal transduction and gene regulation, occur when receptors are ligated or when cells are exposed to HIV proteins or infected with HIV.

Numerous studies have been performed to understand the relationship of HIV infection of the brain and neurological dysfunction. No clear correlation exists between the amount of viral load in the CNS and an individual's likelihood of having HIV encephalitis.[23] Thus, it was proposed that HIV products could participate in the sequelae leading to CNS pathology. Tat is an HIV transactivator protein that is released from HIV-infected cells.[34] It

has been detected in the CNS of individuals infected with HIV, suggesting it to be a possible mediator of damage to the CNS.[29] *In vivo* support for this hypothesis has come from a report showing intraventricular injection of Tat into male rats results in perivascular infiltration of mononuclear cells, gliosis, and apoptosis.[28] Additionally, Tat has been detected in the serum of HIV-infected patients.[31] There are several reports on the *in vitro* effects of Tat. Tat induces MCP-1 expression in astrocytes and in addition, in patients with acquired immune deficiency syndrome dementia, MCP-1 is present in the brain and is elevated in the CSF.[38,45] Tat has also been shown to up-regulate CXCR4 on the surface of resting $CD4^+$ T[33] cells and to mimic the β-chemokines MCP-1, MCP-3, and eotaxin in their interactions with CCR2 and CCR3.[32] Tat can also induce expression of tumor necrosis factor-α (TNF-α) in macrophages and astrocytes as well as infected T cells.[46,47] We and others have shown TNF-α to induce chemokine expression in various CNS cells.[48,49] This indicates that the effects of Tat on chemokine production may be a secondary response of the cell as a result of Tat induction of TNF-α or other cytokines.[36,50] Tat also induces apoptotic cell death in primary human neuronal cultures[37,50] and activates MAP kinase in granular neurons and astrocytes in the rat cerebellum.[51] It induces interleukin-6 in human brain endothelial cells as well as increases adhesion molecule expression.[35,36] In the astrocytic cell line, U-87MG, treatment with Tat leads to increases in HIV-1 gene expression.[35] Astrocytes, despite their inability to produce significant amounts of virus, do produce functional Tat and Rev proteins but not several other key proteins.[52] They suggest that the production of Tat and Rev by astrocytes could contribute to HIV-1 neuropathogenesis. Tat can therefore be produced by both astrocytes and microglia and have potentially widespread effects within the CNS. In this study Tat induced MIP-1α and MIP-1β in astrocytes, but this induction is not statistically significant; however, MCP-1 expression by astrocytes is significantly induced by Tat. Tat also has potent effects on microglia, from which it significantly induces MIP-1α, MIP-1β, and MCP-1. It will be important to analyze the role of Tat in the contest of viral infection in our culture system and to determine whether Tat functions similarly in the presence of other viral factors. Thus, we are currently determining the *in vivo* effects of Tat on chemokine and chemokine receptor expression in the CNS.

Our data demonstrate an autocrine pathway of induction for chemokines. Chemokine autocrine regulation builds on our previous data showing that lipopolysaccharide as well as proinflammatory cytokines can induce chemokines in human fetal microglia as well as astrocytes.[39,49] During an inflammatory response the initial insult may elicit a cascade of cytokines and other cellular factors that may induce chemokines. In more chronic inflammation, high levels of chemokines may facilitate positive-feedback loop-signaling cells to continue producing chemokines, thus perpetuating the inflammatory response. In HIV infection of the CNS, it is possible Tat plays a role in initiating a cascade that results in chemokine autocrine regulation. It is known that HIV-infected individuals who are long-term nonprogressors have elevated levels of circulating chemokines[53,54] and perhaps use this autocrine action of chemokines to maintain such elevated levels.

It is interesting that treatment of microglia with chemokines or Tat does not alter the message levels of their receptors. However, it may be that there are posttranslational alterations. It will be interesting to determine whether there are changes in receptor expression at the surface, ie, internalization or desensitization, whether or not there is a Ca^{2+} flux, and what signal transduction events mediate this phenomenon. Our finding on the expression of chemokine receptors by astrocytes is also intriguing. We find expression of C-C chemokine receptors on astrocytes by immunohistochemistry. In addition, the fact that the astrocytes are responding to chemokines, indicates that there is a receptor on the surface of the cells that recognizes MIP-1α and MIP-1β. We and others[15] have detected the expression of CCR5 in astrocytes by immunohistochemistry and by fluorescence-activated cell sorting (FACS) analysis but there has been no corresponding mRNA data. The lack of expression of specific RNA transcripts for any of the C-C receptors discussed here would suggest that they do not express C-C receptors, CCR1–5. The immunohistochemical and FACS data showing positive staining for CCR5 may indicate cross-reactivity of this antibody for a similar and as yet unidentified receptor on the surface of astrocytes.

The presence of Tat, chemokines, and chemokine receptors in the CNS of patients with HIV encephalitis may result in the inhibition or propagation of the encephalitic process due to interactions among these factors. Further studies both *in vitro* and *in vivo* are necessary to delineate the precise role of these factors in the pathogenesis of HIV encephalitis. In addition, the ability of chemokines to act as autocrine regulators of the local environment of the inflammatory reaction should be considered in the development of therapeutics to either ameliorate or enhance the inflammatory response.

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References

1. Luster A: Chemotactic cytokines in inflammation. N Engl J Med 1998, 338:436–445
2. Rollins B: Chemokines. Blood 1997, 90:909–928
3. Feng Y, Broder CC, Kennedy PE, Berger EA: HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane domain, G-protein coupled receptor. Science 1996, 272:872–877
4. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: CC CKR5: A RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 1996, 272: 1955–1958
5. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA: HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 1996, 381:667–673
6. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L,

Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J: The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996, 85:1135–1148
7. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P: Identification of RANTES, MIP-1α, and MIP-1β as the major HIV suppressive factors produced by $CD8^+$ T cells. Science 1995, 270: 1811–1815
8. Kolson DL, Lavi E, Gonzalez-Scarano F: The effects of HIV in the CNS. Adv Virus Res 1998, 50:1–47
9. Lavi E, Kolson DL, Ulrich AM, Fu L, Gonzalez-Scarano F: Chemokine receptors in the human brain and their relationship to HIV infection. J Neurovirol 1998, 4:301–311
10. Gabuzda D, He J, Ohagen A, Vallat A: Chemokine receptors in HIV-1 infection of the central nervous system. Semin Immunol 1998, 10: 203–213
11. McManus CM, Berman JW, Brett FM, Staunton H, Farrell M, Brosnan CF: MCP-1, MCP-2, and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. J Neuroimmunol 1998, 86:20–29
12. Simpson JE, Newcombe J, Cuzner ML, Woodroofe MN: Expression of monocyte chemoattractant protein-1 and other beta chemokines by resident glia and inflammatory cells in multiple sclerosis. J Neuroimmunol 1998, 84:238–249
13. Sasseville VG, Smith MM, Mackay CR, Pauley DR, Mansfield KG, Ringler DJ, Lackner AA: Chemokine expression in simian immunodeficiency-induced AIDS encephalitis. Am J Pathol 1996, 151:1341–1351
14. Schmidtmayerova H, Nottet HS, Nuovo G, Raabe T, Flanagan CR, Dubrovsky L, Gendelman HE, Cerami A, Bukrinsky M, Sherry B: Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. Proc Natl Acad Sci USA 1996, 93:700–704
15. Rottman JB, Ganley KP, Williams K, Wu L, Mackay CR, Ringler DJ: Cellular localization of the chemokine receptor CCR5. Am J Pathol 1997, 151:1341–1351
16. Vallat A-V, Girolami UD, He J, Mhashilkar A, Marasco W, Shi B, Gray F, Bell J, Keohane C, Smith TW, Gabuzda D: Localization of HIV-1 co-receptors CCR5 and CXCR4 in the brains of children with AIDS. Am J Pathol 1998, 152:167–178
17. Westmoreland SV, Rottman JB, Williams KC, Lackner AA, Sasseville VG: Chemokine receptor expression on resident and inflammatory cells in the brains of macaques with simian immunodeficiency virus encephalitis. Am J Pathol 1998, 152:659–665
18. Xia M, Qin S, Wu L, Mackay C, Hyman BT: Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. Am J Pathol 1998, 153:31–37
19. Hesselgesser J, Horuk R: Chemokine and chemokine receptor expression in the central nervous system. J Neurovirol 1999, 5:13–26
20. Dickson DW, Mattiace LA, Kure K, Hutchins K, Lyman WD, Brosnan CF: Microglia in human disease, with an emphasis on acquired immune deficiency syndrome. Lab Invest 1991, 64:135–156
21. Nath A, Hartloper V, Furer M, Fowke KR: Infection of human fetal astrocytes with HIV-1: viral tropism and the role of cell to cell contact in viral transmission. J Neuropathol Exp Neurol 1995, 54:320–330
22. Dewhurst S, Sakai K, Stevenson M, Evinger-Hodges MJ, Volsky DJ: Persistent productive infection of human glial cells by HIV and by infectious molecular clones of HIV. J Virol 1987, 61:3774–3782
23. Bossi P, Dupin N, Coutellier A, Bricaire F, Lubetzki C, Katlama C, Calvez V: The level of human immunodeficiency virus (HIV) type 1 RNA in cerebrospinal fluid as a marker of HIV encephalitis. Clin Infect Dis 1998, 26:1072–1073
24. Jeang KT: Tat structure and function. Human Retroviruses and AIDS. Edited by G Meyers, LE Henderson, B Korber, KT Jeang, S Wain-Hobson. Los Alamos, Los Alamos National Laboratory, 1996, pp 3–18
25. Tardieu M, Hery C, Peudenier S, Boespflug O, Montagnier L: Human immunodeficiency virus type 1-infected monocytic cells can destroy human neural cells after cell-to-cell adhesion. Ann Neurol 1992, 32: 11–17
26. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfield P, Gallo RC: Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J Virol 1993, 67:277–287
27. Cheng J, Nath A, Knudsen B, Hochman S, Geiger JD, Ma M, Magnuson DS: Neuronal excitatory properties of human immunodeficiency virus type 1 Tat protein. Neuroscience 1998, 82:97–106
28. Jones M, Olafson K, Bigio MRD, Peeling J, Nath A: Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement. J Neuropathol Exp Neurol 1998, 57:563–570
29. Wiley CA, Baldwin M, Achim CL: Expression of HIV regulatory and structural mRNA in the central nervous system. AIDS 1997, 10:843–847
30. Wesselingh S, Levine B, Fox RJ, Choi S, Griffin DE: Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. J Immunol 1994, 152:1289–1297
31. Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH: Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature 1995, 375:497–500
32. Albini A, Ferrini S, Bernelli R, Sforzini S, Giunciuglio D, Aluigi MG, Proudfoot AE, Alouani S, Wells TN, Mariani G, Rabin RL, Farber JM, Noonan DM: HIV-1 Tat protein mimicry of chemokines. Proc Natl Acad Sci USA 1998, 95:13153–13158
33. Secchiero P, Zella D, Capitani S, Gallo RC, Zani G: Extracellular HIV-1 Tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells. J Immunol 1999, 162:2427–2431
34. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B: HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS 1997, 11:1421–1431
35. Zidovetzki R, Wang JL, Chen P, Jeyaseelan R, Hofman F: Human immunodeficiency virus tat protein induces interleukin 6 mRNA expression in human brain endothelial cells via protein kinase C dependent protein kinase pathways. AIDS Res Hum Retroviruses 1998, 14:825–833
36. Sawaya BE, Thatikunta P, Denisova L, Brady J, Khalili K, Amini S: Regulation of TNF-alpha and TGF-beta1 gene transcription by HIV-1 tat in CNS cells. J Neuroimmunol 1998, 87:33–42
37. Shi B, Raina J, Lorenzo A, Busciglio J, Gabuzda D: Neuronal apoptosis induced by HIV-1 tat protein and TNF-alpha: potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia. J Neurovirol 1998, 4:281–290
38. Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, Gallo RC, Major EO: Induction of monocyte chemoattractant protein-1 in HIV-1 tat stimulated astrocytes and elevation in AIDS dementia. Proc Natl Acad Sci USA 1998, 95:3117–3121
39. McManus CM, Brosnan CF, Berman JW: Cytokine-induced MIP-1alpha and MIP-1beta expression in human fetal microglia. J Immunol 1998, 160:1449–1455
40. Karpus WJ, Lukacs NW, McRae BL, Strieter RM, Kunkel SL, Miller SD: An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. J Immunol 1995, 155:5003–5010
41. Karpus WJ, Kennedy KJ: MIP-1alpha and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. J Leukoc Biol 1997, 62:681–687
42. Lee SC, Liu W, Brosnan CF, Dickson DW: Characterization of primary human fetal dissociated central nervous system cultures with an emphasis on microglia. Lab Invest 1992, 67:465–476
43. Ma M, Nath A: Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. J Virol 1997, 71:2495–2499
44. Barnes DA, Huston M, Perez HD: TNF-alpha and IL-1beta cross-desensitization of astrocytes and astrocytoma cell lines. J Neuroimmunol 1998, 87:17–26
45. Cinque P, Vago L, Mengozzi M, Torri V, Ceresa D, Vicenzi E, Transidico P, Vagani A, Sozzani S, Manotvani A, Lazzarin A, Poli G: Elevated cerebrospinal fluid levels of monocyte chemotactic protein-1 correlate with HIV-1 encephalitis and local viral replication. AIDS 1998, 12:1327–1332
46. Buonaguro L, Barillari G, Chang HK, Bohan CA, Cao V, Morgan R, Gallo RC, Ensoli B: Effects of the human immunodeficiency virus type

1 Tat protein on the expression of inflammatory cytokines. J Virol 1992, 66:7159–7167
47. Chen P, Mayne M, Power C, Nath A: The tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. J Biol Chem 1997, 272:22385–22388
48. Oh JW, Schweibert LM, Benveniste EN: Cytokine regulation of CC and CXC chemokine expression by human astrocytes. J Neurovirol 1999, 5:82–94
49. Hurwitz AA, Lyman WD, Berman JW: Tumor necrosis factor alpha and transforming growth factor beta upregulate astrocyte expression of monocyte chemoattractant protein-1. J Neuroimmunol 1995, 57:193–198
50. New DR, Ma M, Epstein LG, Nath A, Gelbard HA: HIV-1 tat protein induces death by apoptosis in primary human neuron cultures. J Neurovirol 1997, 3:168–173
51. Menegon A, Leoni C, Benfenati F, Laltorta F: Tat protein from HIV-1 activates MAP kinase in granular neurons and glial cells from rat cerebellum. Biochem Biophys Res Commun 1997, 238:800–805
52. Gorry PR, Howard JL, Churchill MJ, Anderson JL, Cunningham A, Adrian D, McPhee DA, Purcell DF: Diminished production of human immunodeficiency virus type 1 in astrocytes results from inefficient translation of gag, env, and nef mRNAs despite efficient expression of Tat and Rev. J Virol 1999, 73:352–361
53. Ullum H, Cozzi AL, Victor J, Aladdin H, Phillips AN, Gerstoft J, Skinhoj P, Pedersen BK: Production of beta-chemokines in human immunodeficiency virus (HIV) infection: evidence that high levels of macrophage inflammatory protein-1beta are associated with a decreased risk of HIV disease progression. J Infect Dis 1998, 177:331–336
54. Zagury D, Lachgar A, Chams V, Fall LS, Bernard J, Zagury JF, Bizzini B, Gringeri A, Santagostino E, Rappaport J, Feldman M, O'Brien SJ, Burny A, Gallo RC: C-C chemokines, pivotal in protection against HIV type 1 infection. Proc Natl Acad Sci USA 1998, 95:3857–3861